

Transcription and Translation Inhibitors Permit Metamorphosis up to Radiole Formation in the Serpulid Polychaete *Hydroides elegans* Haswell

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Abstract. Settlement and metamorphosis in most well-studied marine invertebrates are rapid processes, triggered by external cues. How this initial environmentally mediated response is transduced into morphogenetic events that culminate in the formation of a functional juvenile is still not well understood for any marine invertebrate. The response of larvae of the serpulid polychaete *Hydroides elegans* to inhibitors of mRNA and protein synthesis was examined to determine if metamorphosis requires these molecular processes. Competent larvae of *H. elegans* were induced to metamorphose by exposing them to a bacterial film or a 3-h pulse of 10 mM CsCl in the presence of the gene-transcription inhibitor DRB (5,6-dichloro-1- β -D-ribofuranosylbenzimidazole) or the translation inhibitor emetine. When induced to metamorphose in the presence of either inhibitor, larvae of *H. elegans* progressed through metamorphosis to the point at which branchial radioles start to develop. DRB and emetine inhibited the incorporation of radiolabeled uridine into RNA and radiolabeled methionine into peptides, respectively, indicating that they were effective in blocking the appropriate syntheses. Taken together, these results indicate that the induction of metamorphosis in *H. elegans* does not require *de novo* transcription or translation, and that the form of the juvenile worm is achieved in two phases. During the first phase, larvae respond to the inducer by attaching to the substratum, secreting a primary tube, resorbing the prototroch cilia, undergoing caudal elonga-

tion, and differentiating the collar; once the collar is formed, they begin secreting the secondary, calcified tube. During the second phase, the small worm develops branchial radioles and begins to grow, requiring new mRNA and protein syntheses.

Introduction

Complex life histories in which a larval stage undergoes metamorphosis to achieve a juvenile form are common throughout the animal kingdom, including at least 15 phyla of marine invertebrates (Strathmann, 1993). Well-known examples of this postembryonic transformation are amphibians and insects, which have been used as model systems for vertebrates and invertebrates for decades (see: Gilbert *et al.*, 1996; Nijhout, 1999; Rose, 1999). In both groups, the metamorphic process is driven by the action of stage-specific hormones that activate gene expression. In amphibians, thyroid hormones act directly on target tissues by interacting with specific nuclear thyroid-hormone receptors that regulate transcription of target genes containing thyroid-hormone response elements (Evans, 1988; Tata, 1996, 1999). Similarly, metamorphosis in insects is regulated by a complex interaction between a unique group of compounds, the juvenile hormones and ecdysteroids, which also activate transcription factors (see Gilbert *et al.*, 1996).

In contrast, metamorphosis in most well-studied marine invertebrates is set in motion by environmental cues that trigger the developmental events that culminate in the transformation of a larva into a functional juvenile (Hadfield, 2000). This complex set of interactions, from the perception of an inducer signal to the acquisition of the juvenile form, is poorly understood. Some insights into the signal-transduction mechanisms have been gained for different groups,

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Abbreviations: DRB, 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole.

including coelenterates (Freeman and Ridgway, 1990; Schneider and Leitz, 1994; Leitz, 1997; D. W. McCauley, 1997; Thomas *et al.*, 1997; Berking, 1998), polychaetes (Jensen and Morse, 1990; Pawlik, 1990; Ilan *et al.*, 1993; Holm *et al.*, 1998; Biggers and Laufer, 1999), molluscs (Trapido-Rosenthal and Morse, 1985a, b; Baxter and Morse, 1987; Hadfield, 1998; Hadfield *et al.*, 2000; Pires *et al.*, 2000), and crustaceans (Clare *et al.*, 1995; Clare, 1996; Knight *et al.*, 2000). Recent data on the tropical nudibranch *Phestilla sibogae* show that the apical sensory organ in the veliger larvae bears the receptors for the inducer of settlement and metamorphosis (Hadfield *et al.*, 2000). However, little is yet known about how initial inducer-signal transduction mechanisms are coupled with the morphogenetic changes of metamorphosis or to what extent metamorphosis in marine invertebrates requires *de novo* gene expression.

To date, the molecular mechanisms that shape the morphogenetic changes during metamorphosis have been documented in only two aquatic invertebrate phyla. In coelenterates, pattern formation has been analyzed for *Hydra* spp. (summarized by Steele, 2002), and the molecular events during metamorphosis have been documented for *Hydractinia echinata* (summarized by Berking, 1998; Leitz, 1998). Recently, many new signaling molecules that have morphogenetic activity and genes that participate in head formation have been characterized in *Hydra* spp. (see: Broun *et al.*, 2000; Takahashi *et al.*, 2000; Yan *et al.*, 2000a, b; Leontovich *et al.*, 2000). Intensive efforts to understand the molecular events during metamorphosis in molluscs have been made in the nudibranch *Phestilla sibogae* (B. J. McCauley, 1997; Hadfield, 1998; Del Carmen and Hadfield, 1999) and the red abalone *Haliotis rufescens* (Cariolou and Morse, 1988; Degnan and Morse, 1993, 1995; Fenteany and Morse, 1993; Degnan *et al.*, 1995).

In the serpulid polychaete *Hydroides elegans*, most of the morphogenetic changes of metamorphosis are completed during the first 3.5 h after contact with a metamorphic cue contained in bacterial biofilms (Carpizo-Ituarte and Hadfield, 1998; Unabia and Hadfield, 1999). Within 5 min of being exposed to a biofilmed surface, larvae of *H. elegans* begin to crawl upon it and then quickly tether themselves to the surface by a thin mucous thread secreted from their posterior segments. They next lie prone upon the surface and rapidly secrete a primary membranous tube. Within the primary tube, the prototroch is resorbed, the larval body elongates, and the collar region becomes functional. When processes are complete, the small worm begins to secrete the calcified secondary tube, starting from the aperture of the primary tube. Two to three hours after the initiation of settlement, the lobes that will form the branchial radioles become apparent on the head. At this point metamorphosis is complete (Carpizo-Ituarte and Hadfield, 1998). Before the present investigation, attempts to characterize the molecular

mechanisms that regulate metamorphosis had not been reported for a polychaete.

The major questions addressed in this study are, can metamorphosis be triggered in *Hydroides elegans*, and how far can it progress without *de novo* synthesis of mRNA or proteins? *Hydroides elegans* is an excellent model for studies of metamorphosis in marine invertebrates because it has a short generation time (about 21 days), it reproduces year-round in Hawaii, and its metamorphosis may be manipulated by natural and artificial inducers (Hadfield *et al.*, 1994; Carpizo-Ituarte and Hadfield, 1998; Holm *et al.*, 1998; Unabia and Hadfield, 1999). The results of the present study show the extent to which syntheses of RNA and protein are involved in the progression of metamorphosis in *Hydroides elegans*.

Materials and Methods

Collection of adults and culture of larvae

Adults of *Hydroides elegans* were collected from Pearl Harbor, Hawaii. Gametes were collected, and competent larvae were reared by methods described previously (Hadfield *et al.*, 1994; Carpizo-Ituarte and Hadfield, 1998). Competence is defined for this species as the ability of larvae to settle and metamorphose when exposed to a well-developed marine biofilm. Larvae attained metamorphic competence as three-segmented nectochaetes about 4–5 days after fertilization (at 25–26 °C).

Effects of 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB) and emetine

Assays for metamorphosis, using 5–6-day-old competent larvae, were conducted in 5-ml petri dishes containing natural seawater that had been passed through a 0.22- μ m filter (FSW). Four replicates per treatment were used in all experiments.

To examine the effects of inhibitors of transcription (DRB) or translation (emetine) on the ability of larvae to complete metamorphosis, 30–90 competent larvae were added to each dish containing one of the inhibitors. To induce metamorphosis, the larvae were exposed to a marine biofilm or a 3-h pulse of 10 mM CsCl in the presence of one of the inhibitors. Two types of experiments were performed. In one, larvae were exposed to the inducer (marine biofilm or CsCl) and the inhibitor (DRB or emetine) simultaneously. In the second type, larvae were preincubated in DRB for 1–12 h or in emetine for 1–2 h before inducing them to metamorphose in the presence of the inhibitor. The number of larvae that had metamorphosed was determined between 4 and 24 h after initial induction by observing them with a dissecting microscope. Larvae were considered to have metamorphosed when they had elongated, resorbed the prototroch, and secreted the primary and secondary (calcar-

eous) tubes. At higher concentrations of inhibitors, the branchial radioles did not differentiate, and the newly metamorphosed worms were transferred from the solution with the inhibitor to fresh FSW and observed for an additional 16–24 h.

In all experiments, either pieces of plastic mesh or petri dishes that had accumulated a marine biofilm while soaking in the laboratory seawater tables for several days were used as a positive control to verify that the larvae were metamorphically competent. A negative control, included to measure spontaneous metamorphosis, consisted of placing larvae in FSW in clean plastic petri dishes during the experimental period.

Stock solutions of emetine (Sigma Chemical Co.) and DRB (Calbiochem) were prepared in 100% ethanol (ETOH) and added to filtered seawater to desired concentrations. Controls were included in each experiment to detect possible effects of ETOH on metamorphosis.

Labeling of RNA and protein synthesis "in vivo"

To confirm that DRB and emetine were effectively inhibiting RNA and protein synthesis, respectively, the synthesis of these macromolecules was measured *in vivo* in the presence and absence of the inhibitors. All of the experiments were conducted in 1.5-ml microcentrifuge tubes (Eppendorf) using artificial seawater (ASW) (Cavanaugh, 1956) containing antibiotics (0.1 mg/l penicillin; 0.1 mg/l streptomycin).

Two experiments were carried out to measure the effects of DRB. In the first, 400–1000 larvae were incubated for 2.25 h with 10, 30, 50, or 70 μM DRB, and then 0.3 μl (0.3 μCi) of [5,6- ^3H]uridine (Amersham, 38 Ci/mmol) was added for the last 45 min of incubation in the inhibitor. In the second experiment, 400–1000 larvae were exposed to 10 μM DRB for 3, 6, 12, or 24 h, and then 3 μl (0.3 μCi) of [5,6- ^3H]uridine was added during the last 45 min of incubation in DRB. The labeling was stopped by centrifuging the tubes (15,120 $\times g$, 5 min) at room temperature (RT = 25 $^{\circ}\text{C}$) and then removing the supernatant fluid and immersing the pelleted larvae, still in the tubes, in liquid nitrogen. The pellets were homogenized on ice and resuspended in 1 ml of SSC buffer (sodium chloride/sodium citrate, pH 7.0) prior to trichloroacetic acid (TCA) precipitation and protein-concentration analysis. TCA precipitation of RNA was carried out according to the protocol described by Fenteany and Morse (1993). Absolute TCA was added to 800 μl of resuspended sample (to a final concentration of 10% TCA). Immediately afterward, the sample was vortexed and allowed to precipitate on ice for 30 min. The precipitate was collected on a microfiber filter (Gelman, Type E), washed three times with 5% TCA and twice with 100% ETOH, and dried for an hour at RT. The filters were introduced to vials with 5 ml of scintillation

fluid (ScintiSafe, Econo 2, Fisher Scientific), and radioactivity was measured in a Beckman LS 7000 liquid scintillation system or a Packard liquid scintillation analyzer 1900 TR. The remaining 200 μl from the initial samples was used to measure protein concentrations by the Bradford method (Ausubel *et al.*, 1992).

Four experiments were carried out to measure the effect of emetine on metamorphosis. In each of the experiments, 400–1000 competent larvae were used. In the first experiment, larvae were incubated for 4 h in 0, 250, 500, and 1000 nM emetine, and 1 μl (0.01 mCi) of ^{35}S -methionine (Amersham, 10 mCi/ml) was added during the last 1.5 h of incubation in the inhibitor. In a second experiment, larvae were incubated in 250 nM emetine for 0, 6, and 9 h before adding 1 μl (0.01 mCi) of ^{35}S -methionine (Amersham, 10 mCi/ml) during the last 2 h of incubation. That is, for the treatment of 0 h, emetine was added simultaneously with ^{35}S -methionine and incubated for 2 h. In a third experiment, the effect of different periods of incubation in 1 μM emetine was measured. Larvae were incubated for 1, 2, 4, and 6 h in 250 nM emetine, and 1 μl (0.01 mCi) of ^{35}S -methionine was added to the emetine-FSW solution for an additional 45 min. Larvae in the treatment of 1-h emetine remained a total of 1 h and 45 min in emetine, but were only in the presence of both emetine and ^{35}S -methionine for the last 45 min. During the fourth experiment, the effect of 250 nM emetine was measured when larvae were induced to metamorphose with a 3-h pulse of 10 mM CsCl. During this experiment, larvae were placed for 1 or 2 h in 250 nM emetine before being exposed to a 3-h pulse of CsCl (10 mM) in the emetine-FSW solution. One microliter (0.01 mCi) of ^{35}S -methionine was added during the last 1.5 h of the Cs $^{+}$ pulse. After the period of incubation in ^{35}S -methionine, the larvae were centrifuged (15,120 $\times g$, 2 min), and a 50- μl sample of the supernatant fluid was recovered to measure the radioactivity remaining in it. The remaining supernate was removed from the tube, and the larvae were washed once with ASW and resuspended in 500 μl of 50 mM sodium phosphate buffer, pH 7.2. The larval tissue was lysed by sonication for 30 s in a Branson Sonifier (model 450, Branson Ultrasonics Corporation). The sample was removed from the sonicator every 10 s and put on ice for 20 s to prevent overheating of the tissue. TCA precipitation was carried out according to the protocol described by Ausubel *et al.* (1987) to measure incorporation of ^{35}S -methionine into newly synthesized proteins. The sonicated sample was centrifuged (15,120 $\times g$, 2 min) at RT, and the supernatant fluid was transferred to a clean 1.5-ml microtube (Eppendorf). To a 50- μl sample of this supernate was added 0.5 ml of bovine serum albumin (0.1 mg/ml) containing 0.02% NaN_3 followed by 0.5 ml of ice-cold 20% TCA, and the suspension was incubated on ice for 30 min. After incubation, the suspension was filtered under vacuum onto a microfiber filter (Gelman, Type E), washed twice with ice-cold

10% TCA and twice with 100% ETOH, and the filters were allowed to dry for 30–45 min at RT. To quantify the incorporation of ^{35}S -methionine, radioactivity was measured by introducing the fiberglass filters with the TCA precipitate into vials containing 5 ml of scintillation fluid (ScintiSafe, Econo 2; Fisher Scientific). Radioactivity in the vials was measured in a Beckman LS 7000 liquid scintillation system or a Packard liquid scintillation analyzer 1900 TR. Results were expressed as DPM/ μg of protein. The protein concentration in the remaining sample (250–450 μl) was measured using the spectrophotometric method described by Whitaker and Granum (1980).

Statistical analysis of metamorphosis

The proportion of larvae that underwent metamorphosis was determined, and these values were arcsine-transformed to estimate statistical differences among treatments using one-way ANOVAs or Kruskal-Wallis ANOVAs of ranks when equal-variance tests failed. Pairwise multiple comparisons were tested using the Student-Newman-Keuls test or Dunnett's method when all treatments were compared to each other, or Bonferroni's method when treatments were compared to a control. All statistical tests were conducted with the aid of SigmaStat software (SPSS Inc.).

Results

Effects of the transcription inhibitor DRB on metamorphosis

When competent larvae of *Hydroides elegans* were induced to metamorphose with a bacterial biofilm or a 3-h pulse of CsCl in the presence of DRB, they completed metamorphosis of the larval body after 3–4 h but did not develop branchial radioles (Fig. 1). The minimum effective concentration of DRB to prevent branchial radiole formation in all the larvae tested with either inducer was 10 μM (Figs. 2, 3; Bonferroni test, $P < 0.05$). Lower concentrations of DRB tested either had no effect (0.1 μM , Figs. 2, 3) or showed only a slight, but significant (Bonferroni test, $P < 0.05$), reduction in the proportion of larvae that completed formation of branchial radioles (1 μM , Fig. 2).

Preincubation of competent larvae of *H. elegans* in 10 μM DRB for up to 9 h before addition of an inducer (biofilm or CsCl) did not inhibit metamorphosis (Figs. 4, 5). In fact, larvae responded significantly faster (Student-Newman-Keuls test, $P < 0.05$) to biofilm in the presence of DRB than in its absence (Fig. 4A), a difference that was reduced after 14 h of incubation when the experiment was stopped (Fig. 4B).

Preincubation in 10 μM DRB for up to 9 h did not stop the larvae from metamorphosing in response to a pulse of CsCl, but a significant reduction in the percentage that

metamorphosed was observed (Bonferroni's method, $P < 0.05$, Fig. 5).

Effects of the protein-synthesis inhibitor emetine on metamorphosis

Competent larvae of *H. elegans* metamorphosed when exposed to a biofilm in the presence of 1 μM emetine (Fig. 6), with no significant reduction in the proportion of larvae that metamorphosed after 3 h compared with induction by a biofilm alone. The larvae progressed in metamorphosis to the point at which branchial radioles begin to develop; concentrations of emetine as low as 250 nM were effective in preventing the formation of these structures. Preincubation for 1 h in 1 μM emetine prior to exposure to a biofilm did not curtail metamorphosis (Fig. 7), but the proportion of larvae that had metamorphosed after 3 h was slightly reduced.

Results similar to the ones observed when a biofilm was used as inducer were obtained when larvae were stimulated to metamorphose with a 3-h pulse of 10 mM CsCl in the presence of emetine (Fig. 8). The proportion of larvae that metamorphosed was similar in the presence and absence of 1 μM emetine, and the proportions that metamorphosed in these treatments were similar to those obtained in response to a bacterial biofilm.

Labeling RNA and proteins in vivo

Incorporation of [5,6- ^3H]uridine into RNA by competent larvae of *H. elegans* in the presence of DRB varied with concentration and period of exposure. In comparison to incorporation without an RNA-synthesis inhibitor, [5,6- ^3H]uridine incorporation was reduced up to 80% after an incubation period of 2.25 h in 70 μM DRB (Fig. 9A). Even with the lowest concentration of DRB tested (10 μM), incorporation of [5,6- ^3H]uridine was reduced by 60% (Fig. 9A).

When larvae were exposed to 10 μM DRB for 24 h before addition of [5,6- ^3H]uridine, incorporation of [5,6- ^3H]uridine was reduced up to 88% in comparison to that in larvae incubated in the absence of DRB (Fig. 9B). A substantial decrease in incorporation of [5,6- ^3H]uridine, up to 42%, was evident after the first 3 h of exposure to DRB (Fig. 9B). Spontaneous metamorphosis was 3.2% and 6% in the two experiments in control larvae kept in conditions equivalent to those of larvae incubated in the presence of DRB.

Synthesis of new proteins in larvae of *H. elegans* exposed to emetine was dependent on the concentration and length of exposure to the inhibitor. When larvae were exposed to different concentrations of emetine for 4 h, a decrease in ^{35}S -methionine incorporation was observed. A reduction of 41% in incorporation of ^{35}S -methionine into proteins

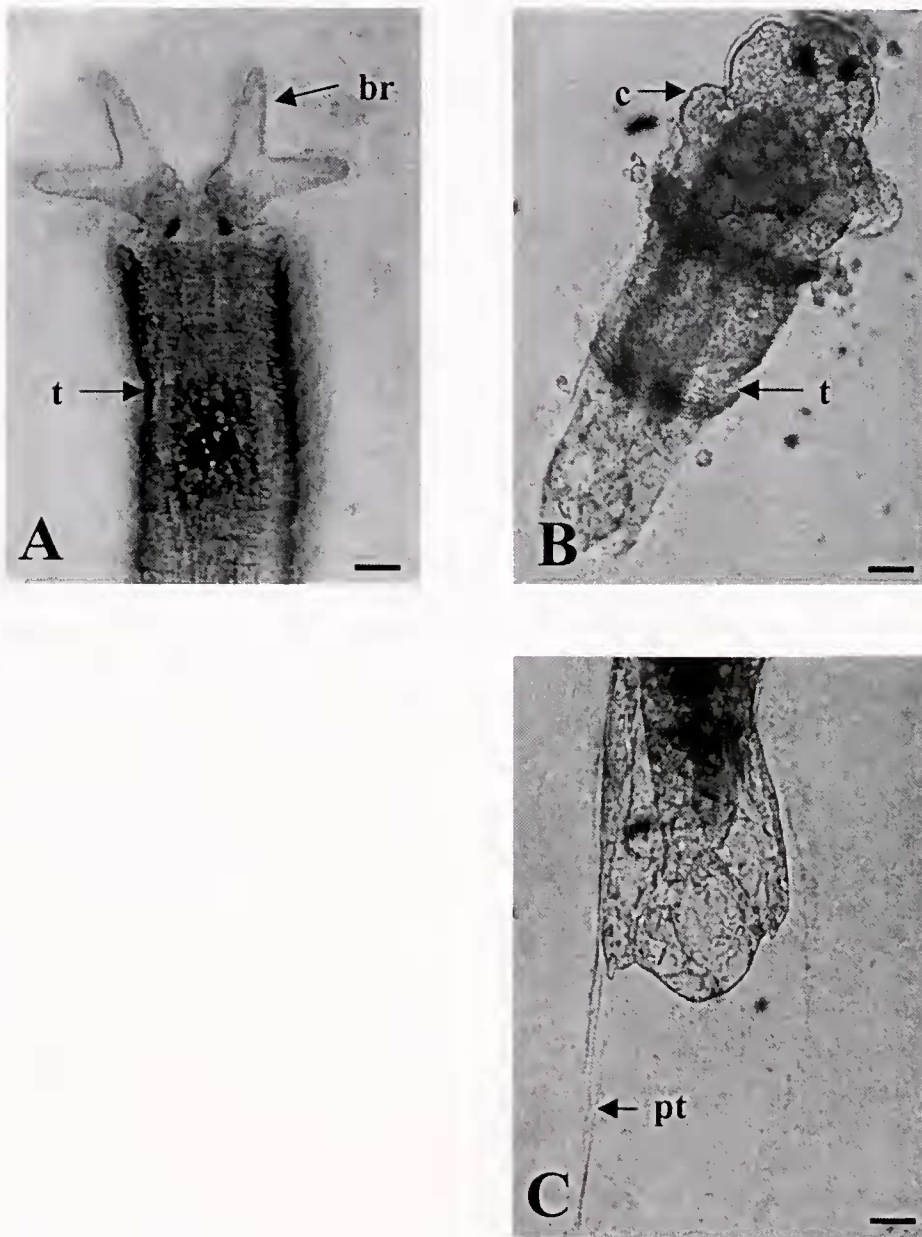


Figure 1. Metamorphosis in *Hydris elegans* in the absence or presence of an inhibitor of macromolecular synthesis. (A) A worm newly metamorphosed in response to a bacterial biofilm. (B) A worm in which metamorphosis was induced in the presence of the translation inhibitor emetine. (C) The posterior section of the tube of the worm shown in B. Newly metamorphosed worms exposed to induction cues in the presence of the transcription inhibitor DRB have the morphology shown in B. br, branchial radioles; c, collar; pt, primary tube; t, secondary calcareous tube. Scale bar = 20 μ m for all micrographs.

occurred with exposure to 250 nM emetine, and a reduction of up to 87% occurred in response to 1 μ M emetine (Fig. 10A). Exposure of larvae to 1 μ M emetine for 1 h was sufficient to reduce incorporation of 35 S-methionine into newly synthesized protein by 79%. An increase in the length of exposure to 1 μ M emetine did not substantially reduce the incorporation of the radiolabeled amino acid. A maxi-

mum reduction of 81% in 35 S-methionine incorporation was detected after 6 h of exposure to 1 μ M emetine (Fig. 10B).

An increase in the length of exposure of larvae to 250 nM emetine resulted in lower levels of incorporation of 35 S-methionine. Periods of exposure to the inhibitor of 6 and 9 h reduced 35 S-methionine incorporation by 68% and 74% respectively, in comparison to controls where no emetine

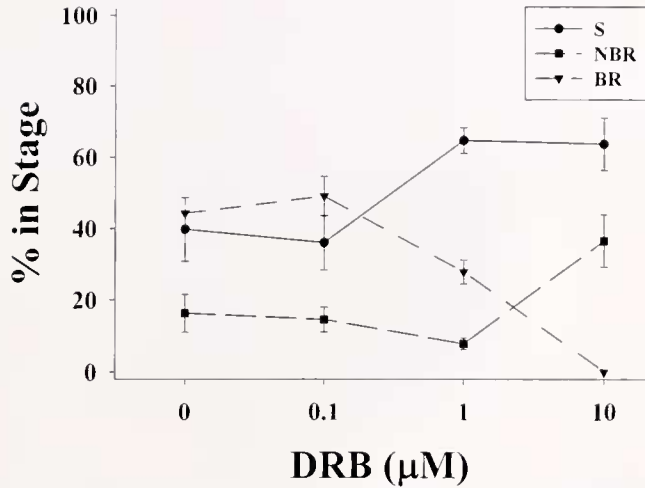


Figure 2. Effects of the transcription inhibitor DRB on metamorphosis and differentiation of branchial radioles in *Hydroides elegans*. Larvae were induced to metamorphose with a marine biofilm in the presence (0.1, 1, and 10 μM) and absence (0) of DRB for 3.5 h. Points indicate mean percentages of larvae ± 1 SE (*n* = 4 replicates/treatment) that were swimming (S), metamorphosed without branchial radioles (NBR), or metamorphosed with branchial radioles (BR) at the end of the experiment.

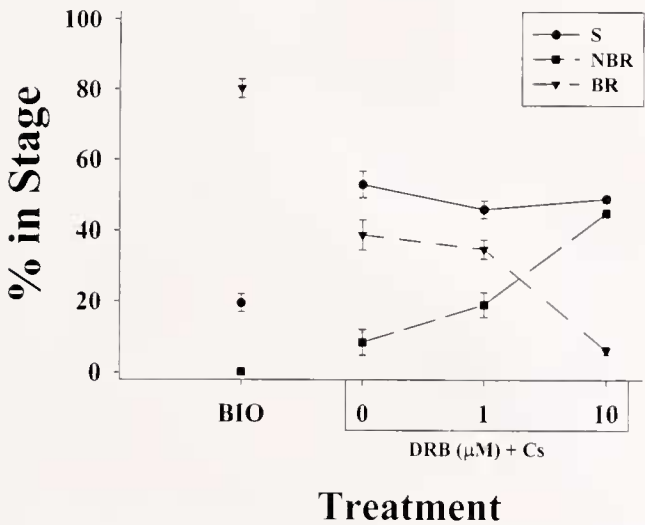
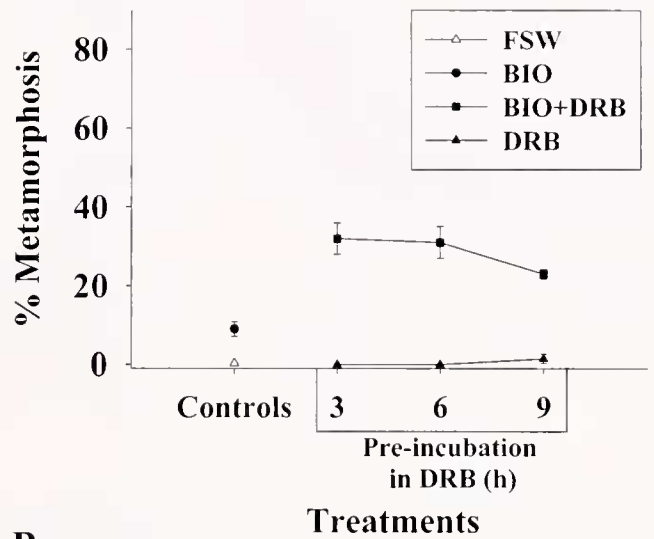


Figure 3. Effects of the transcription inhibitor DRB on metamorphosis and differentiation of branchial radioles in *Hydroides elegans*. Larvae were exposed to a 3-h pulse of 10 mM CsCl in the presence (treatments 1 and 10) and absence (treatments 0 and BIO) of DRB. After the cesium pulse, the solution was replaced with fresh filtered seawater (FSW) containing 1 or 10 μM DRB in the treatments where DRB was included, or only FSW in the treatments where DRB was absent. Points indicate mean percentages of larvae ± 1 SE (*n* = 4 replicates/treatment) that were swimming (S), metamorphosed without branchial radioles (NBR), or metamorphosed with branchial radioles (BR) 24 h after initiation of the cesium pulse.

was present (Fig. 11A). In contrast, the lowest level of incorporation of ³⁵S-methionine into newly synthesized protein (97% reduction) was observed when the larvae were

A.



B.

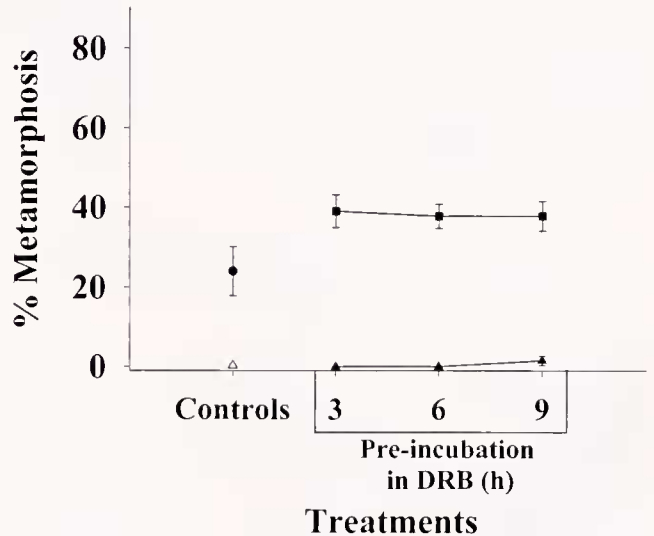


Figure 4. Effects of preincubation in DRB on metamorphosis of *Hydroides elegans*. Larvae were preincubated in 10 μM DRB for 3, 6, or 9 h before transfer to a biofilmed petri dish. In the treatments with DRB, the inhibitor was present throughout the experiment. Points indicate percentages of larvae that metamorphosed ± 1 SE (*n* = 4 replicates/treatment) after being transferred to the petri dish. (A) 3 h after transfer to the inducing biofilm, larvae had metamorphosed to the pre-radiole stage in the treatments where DRB was present. (B) 14 h after transfer to the biofilmed dish, small branchial radioles began to develop in the treatment where DRB was present. BIO, substratum coated with a marine biofilm; FSW, seawater filtered through a 0.22-μm filter; in DRB control, no biofilm was added.

induced with CsCl after being incubated for 2 h in 250 nM emetine (Fig. 11B). Interestingly, induction of larvae to metamorphose with CsCl alone showed a reduction in

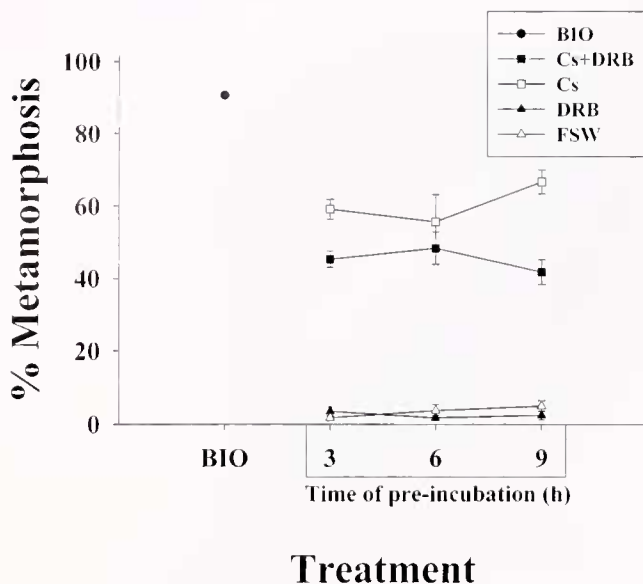


Figure 5. Effects of preincubation in DRB on metamorphosis of *Hydroides elegans*. Larvae were preincubated in 10 μM DRB for 3, 6, or 9 h before being induced to metamorphose with a 3-h pulse of filtered seawater (FSW) containing 10 mM CsCl and 10 μM DRB. After the CsCl pulse, solutions were replaced with FSW, and metamorphosis was allowed to progress for 21 h. Points indicate mean percentages of larvae that metamorphosed ± 1 SE ($n = 4$ replicates/treatment) 24 h after initial exposure to CsCl. DRB, 10 μM DRB; Cs, 10 mM CsCl/ 3 h; BIO, substratum coated with a marine biofilm; FSW, seawater filtered through a 0.22- μm filter. In the treatment where Cs was present with DRB (DRB + Cs), larvae metamorphosed to the pre-radiole stage.

incorporation of ^{35}S -methionine of 82% compared to a control where no CsCl or emetine were present (Fig. 11B).

Discussion

Results from the present study demonstrate two important elements of metamorphosis in *Hydroides elegans*. First, settlement, attachment, secretion of the primary and secondary tubes, velar loss, collar formation, and caudal elongation proceed in the presence of inhibitors that drastically reduce transcription and translation. Second, in the presence of transcription and translation inhibitors, metamorphosis stops at the point when branchial radioles begin to grow.

When competent larvae of *H. elegans* were induced to metamorphose with a bacterial biofilm or CsCl in the presence of effective concentrations of DRB, they completed metamorphosis to the point at which branchial radioles begin to develop. Development of the radioles can be considered the initiation of early juvenile development in *H. elegans*, after all larval structures have been lost and the juvenile shape has been realized. These changes include loss of the prototroch, differentiation of the collar, caudal elongation, and secretion of primary and secondary tubes. Similar results were reported initially for the tropical nudibranch *Phestilla sibogae* by Hadfield (1978) and later confirmed in the same species by B. J. McCauley (1997) and Del Carmen and Hadfield (1999, 2000). In the presence

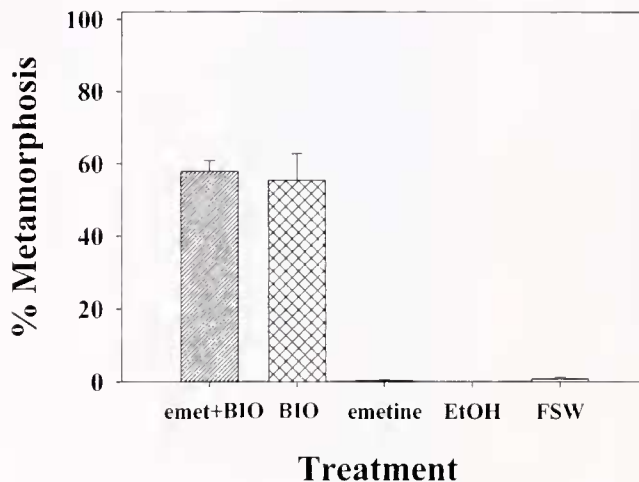


Figure 6. Effects of the translation inhibitor emetine on metamorphosis in *Hydroides elegans*. Larvae were exposed to a marine biofilm in the presence (emet + BIO) or absence (BIO) of 1 μM emetine. Bars indicate percentages of larvae that metamorphosed ± 1 SE ($n = 4$ replicates/treatment) after 3 h of exposure to the biofilm. BIO, substratum coated with a marine biofilm; emetine, emetine 1 μM control; EtOH, equivalent concentration of ethanol used to dissolve emetine; FSW, seawater filtered through a 0.22- μm filter. In the treatment where the biofilm was present with emetine (emet + BIO), larvae metamorphosed to the pre-radiole stage.

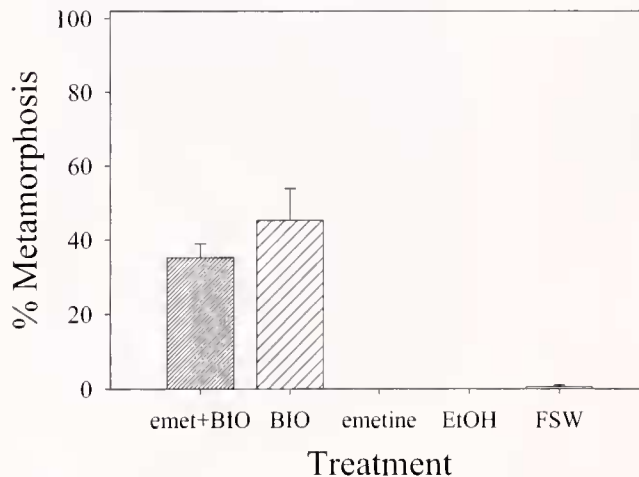


Figure 7. Effects of preincubation in the translation inhibitor emetine on metamorphosis in *Hydroides elegans*. Larvae were incubated for 1 h in 1 μM emetine in a clean petri dish and then transferred to a biofilm-coated petri dish, still in the presence of emetine (emet + BIO). Bars indicate percentages of larvae that metamorphosed ± 1 SE ($n = 4$ replicates/treatment) after 3 h of exposure to the biofilm. Emetine, emetine 1 μM control; EtOH, equivalent concentration of ethanol used to dissolve emetine; FSW, seawater filtered through a 0.22- μm filter. In the treatment where the biofilm was present with emetine (emet + BIO), larvae metamorphosed to the pre-radiole stage.

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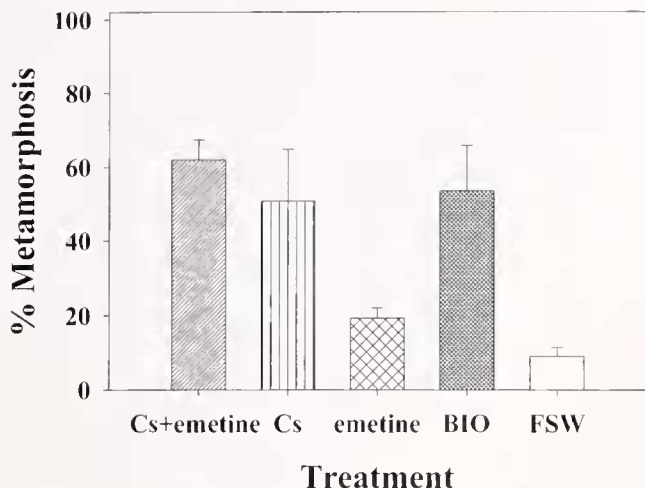


Figure 8. Effects of the translation inhibitor emetine on induction of metamorphosis in *Hydroides elegans*. Larvae were incubated for 3 h in 1 μM emetine in filtered seawater (FSW) that also contained 10 mM CsCl to induce metamorphosis. Solutions were replaced with FSW after 3 h, and the larvae were allowed to complete metamorphosis for another 16 h. Bars indicate percentages of larvae that metamorphosed ± 1 SE ($n = 4$ replicates/treatment). Cs, 10 mM CsCl/3 h; emetine, 1 μM emetine; BIO, substratum coated with a marine biofilm; FSW, seawater filtered through a 0.22- μM filter.

of DRB, transcription and translation were drastically reduced without inhibiting induction of metamorphosis in competent larvae of *P. sibogae*. Further experiments showed that activation of the receptor for the metamorphic signal is not affected by reduction in transcription, but new transcription appears to be necessary for the final, elongation phase of metamorphosis in *P. sibogae* (Del Carmen and Hadfield, 2000).

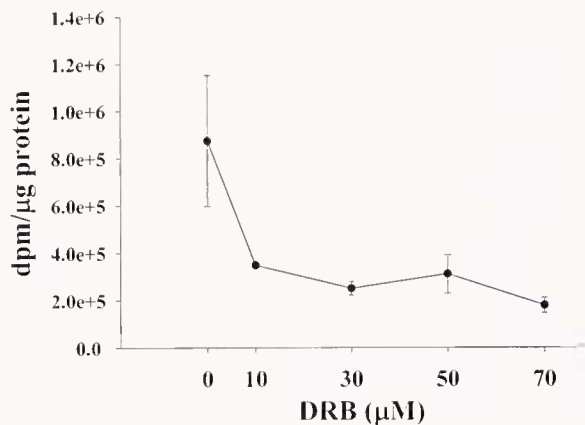
The observation that metamorphosis in *H. elegans* progresses in the presence of DRB, together with the demonstration that DRB effectively inhibits RNA synthesis, indicates that the initiation and early phases of metamorphosis in this species are independent of new RNA synthesis. However, the fact that we were never able to completely inhibit RNA synthesis leaves open the possibility that synthesis of some mRNAs was resistant to the action of DRB, and thus that new transcripts may play an essential role during metamorphosis for the first hours after induction. Transcriptional resistance to DRB in HeLa cells was reported by Zandomeni *et al.* (1982).

The observation that development in the presence of DRB stops at the point when branchial radiole development begins indicates that new transcripts are necessary for the growth of the radioles and that these transcripts are among the more than 80% of RNA syntheses inhibited by DRB. Even a 40% reduction in synthesis of RNA, measured by incorporation of [5,6- ^3H]uridine, was sufficient to prevent the formation of branchial radioles. It is not surprising that active growth of new structures, such as the branchial radi-

oles, is dependent on transcription, because a broad spectrum of new tissues—including nerves, muscles, and secretory epithelium—must be generated, each requiring many new proteins. Experiments on the hydrozoan *Hydractinia echinata* revealed that morphogenesis of the adult polyp can be influenced by pulse applications of α -amanitin or cordycepin. These compounds, which block mRNA transcription at different levels, inhibit the formation of tentacles and stolons in *H. echinata* when applied during late gastrulation or 3 h after induction of metamorphosis (Eiben, 1982).

When larvae of *Hydroides elegans* were induced to meta-

A.



B.

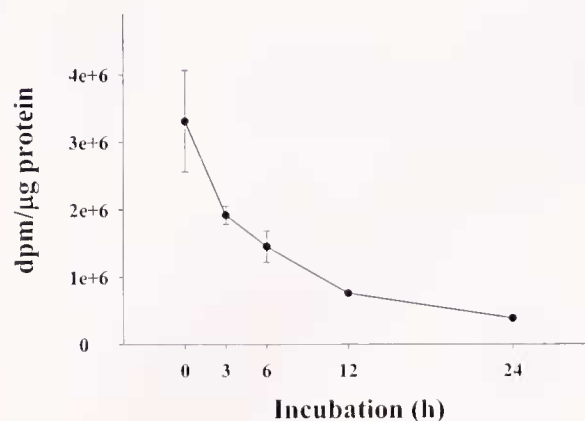
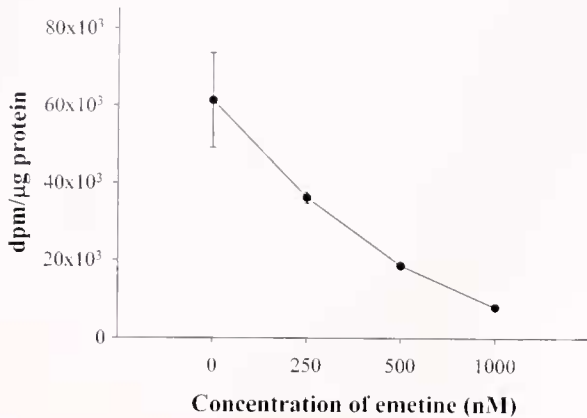


Figure 9. Incorporation of [5-6 ^3H]uridine into newly synthesized RNA by larvae of *Hydroides elegans*. In (A), larvae were incubated for 2.15 h in the presence of the different concentrations of DRB indicated on the x axis. In (B), larvae were incubated in 10 μM DRB in FSW for the different periods of exposure indicated on the x axis. In both (A) and (B), larvae were allowed to incorporate [5-6 ^3H]uridine during the last 45 min. Points are means of uridine incorporation ± 1 SE ($n = 2$ replicates/treatment).

A.



B.

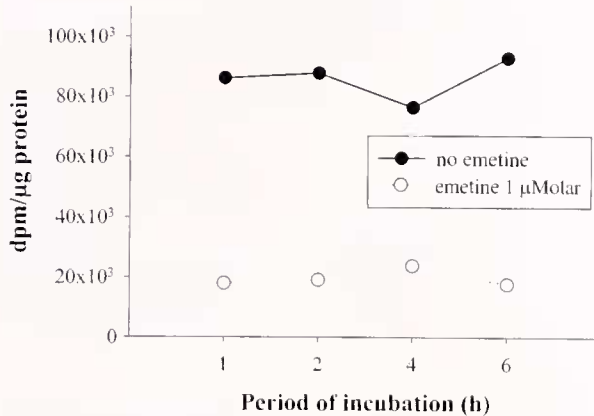
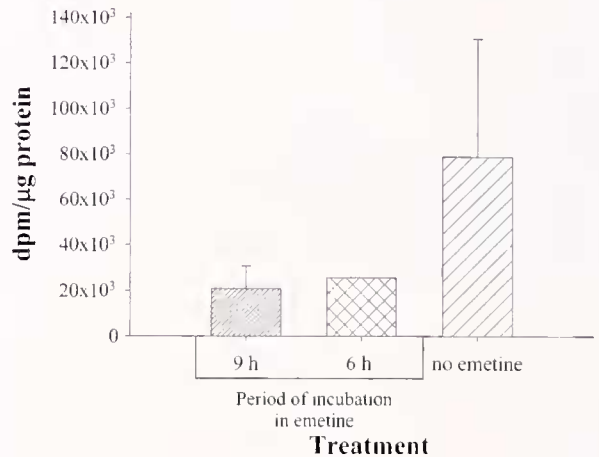


Figure 10. Incorporation of ^{35}S -methionine into newly synthesized proteins by larvae of *Hydroides elegans* in the presence of the translation inhibitor emetine. In (A), larvae were incubated in varying concentrations of emetine, shown on the x axis, for 4 h with incorporation of ^{35}S -methionine during the last 1.5 h. Points are means of methionine incorporation ± 1 SE ($n = 2$ replicates/treatment). In (B), larvae were incubated in $1 \mu\text{M}$ emetine for the intervals indicated on the x axis and allowed to incorporate ^{35}S -methionine for an additional 45 min. Points are mean methionine incorporation ± 1 SE.

morphose with either a bacterial biofilm or a pulse of CsCl in the presence of the translation inhibitor emetine, just as with DRB, they completed metamorphosis and stopped developing before the outgrowth of the branchial radioles. Neither the highest concentration of emetine used ($1 \mu\text{M}$) nor an extended preincubation period in emetine stopped initiation of metamorphic morphogenesis in response to a bacterial biofilm or cesium as inducers. These results are consistent with the hypothesis that initiation of metamorphosis in *H. elegans* is also highly independent of synthesis of new proteins, a result that is concordant with previous studies of other species, especially certain molluscs (Fenteany and Morse, 1993; Del Carmen and Hadfield, 1999).

Larvae of the archaeogastropod *Haliotis rufescens* (Fenteany and Morse, 1993) and the nudibranch *Phestilla sibogae* (B. J. McCauley, 1997; Del Carmen and Hadfield, 1999) initiate metamorphosis in the presence of translation inhibitors. When competent larvae of *H. rufescens* were induced to metamorphose in the presence of concentrations of emetine or anisomycin sufficient to block protein synthesis, settlement and plantigrade attachment still occurred, indicating that these premetamorphic processes do not require *de novo* protein synthesis (Fenteany and Morse, 1993). The authors suggest that the induction of settlement

A.



B.

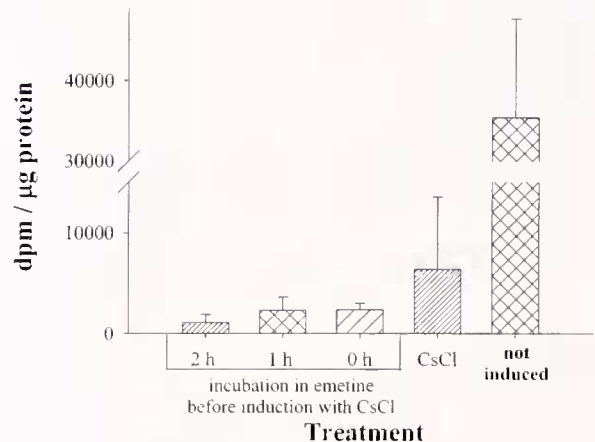


Figure 11. Incorporation of ^{35}S -methionine into newly synthesized proteins by larvae of *Hydroides elegans* after different periods of incubation in 250 nM emetine. In (A), larvae were incubated in emetine at the concentrations indicated on the x axis and allowed to incorporate ^{35}S -methionine during the last 2 h. Points are means of methionine incorporation ± 1 SE ($n = 2$ replicates/treatment, except for 6 h, which represents 1 sample). In (B), larvae were first incubated in $1 \mu\text{M}$ emetine in filtered seawater (FSW) before being induced to metamorphose with a 3-h pulse of CsCl (10 mM) and allowing larvae to incorporate methionine for 45 min after the CsCl pulse and in the presence of emetine. Points are mean methionine incorporation ± 1 SE ($n = 4$ replicates/treatment for 2, 1, and 0 h, and 2 replicates/treatment for CsCl and not induced (no CsCl)).

and plantigrade attachment in the presence of protein-synthesis inhibitors is consistent with the notion that these behavioral responses are controlled by chemosensory mechanisms mediated by the nervous system. Similar results were reported for the larvae of *P. sibogae*: experiments with emetine showed that metamorphosis can be initiated in the presence of the translation inhibitor and that development stops just before final elongation of the body (B. J. McCauley, 1997).

It is particularly interesting that protein synthesis in larvae of *Hydroides elegans* was reduced even further when CsCl was added to induce metamorphosis (Fig. 11B). Kroiher *et al.* (1991) also reported decreased incorporation of amino acids into proteins when larvae of the hydrozoan *Hydractinia echinata* were exposed to CsCl, attributing it to a reduction in the Na⁺ concentration of the medium when CsCl is added. Transport of amino acids across the cell membrane is known to be a function of a Na⁺-transport in marine invertebrates (Stephens, 1988).

Inhibition of development of the branchial radioles by emetine at concentrations at which protein synthesis was reduced by only 40% is consistent with the data obtained with DRB. Thus, unsurprisingly, both new mRNA transcripts and their translation products are necessary for production of the branchial radioles.

Taken together, the data on RNA and protein synthesis in metamorphosing *Hydroides elegans* strongly support the conclusion that metamorphosis and juvenile development occur in two phases—one independent of new gene transcription and translation, and a second in which these two molecular processes are necessary for development and growth to continue. During the first phase, larvae respond rapidly to the bacterial inducer, change their swimming behavior, attach, secrete primary and secondary tubes, lose the prototroch cilia, differentiate the collar, and elongate the caudal region (Carpizo-Ituarte and Hadfield, 1998). During the second phase, the metamorphosed worm develops branchial radioles, a process that requires new RNA and protein synthesis. Results reported for larvae of other marine invertebrates reinforce the idea that metamorphic competence in many marine invertebrate larvae is a developmental stage primed to metamorphose and in which the activation and near completion of metamorphosis requires neither *de novo* synthesis of mRNA nor proteins (Hadfield, 2000).

Since larvae of *H. elegans* survive through metamorphosis with very reduced synthesis of new mRNAs or proteins, cell proliferation, which requires both processes, probably does not play an essential role during metamorphosis. Previous studies in other invertebrate species are consistent with this hypothesis. In *Phestilla sibogae*, labeling of dividing cells with an antibody against a proliferating cell nuclear antigen (PCNA) decreases when larvae reach competency and is detected again only after the larvae have completed

metamorphosis and begun early juvenile development (B. J. McCauley, 1997). In embryos of *Hydractinia echinata*, the index of cell proliferation measured by the BrdU method decreased to 4% by 78 h after fertilization. In larvae of *H. echinata* kept at low temperatures, the cell proliferation index went to zero after 60 to 70 days without affecting the ability of the larvae to undergo metamorphosis (Plickert *et al.*, 1988). Preliminary observations on *Hydroides elegans* using PCNA indicated that cell proliferation decreases sharply when larvae become competent and increases notably in the developing branchial radioles of newly settled juveniles (Carpizo-Ituarte, unpubl. results).

Metamorphosis in most well-studied insects and amphibians is a relatively slow process wherein hormonal transcription factors mediate essential cascades of “*de novo*” transcription and translation to regulate metamorphic morphogenesis (Gilbert *et al.*, 1996; Tata, 1996, 1999; Shimizu-Nishikawa *et al.*, 2002). In contrast, the experimental results obtained with the polychaete *Hydroides elegans* support a generalization noted by Hadfield *et al.* (2001): for most well-studied marine invertebrates, the competent larva carries within it a well-formed juvenile body, which metamorphosis need only liberate from larval structures. In this case, metamorphosis should require little or no *de novo* transcription or translation, processes that will be necessitated when juvenile growth begins. This may be the result of adaptation to undergo rapid metamorphosis and make an extremely vulnerable period—that of a planktonically adapted larva residing on the benthos—as brief as possible (Hadfield, 2000).

The more precisely we define the molecular events of metamorphosis in *Hydroides elegans* and other marine invertebrates, the better we are able to make comparisons with model systems among insects and amphibians. So far, the capacity for metamorphosis to proceed almost independently of new gene expression in many marine invertebrates points toward different mechanisms to initiate postlarval development. To what extent these mechanisms are different from those known in insects and amphibians awaits further investigation. Knowledge of the signal-transduction pathways that are activated during induction of metamorphosis and how the initial triggering event orchestrates the genetic machinery to turn a larva into a juvenile in a wide variety of marine invertebrates will help us to understand how the widespread phenomenon of metamorphosis in marine invertebrates evolved.

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